

***dead end*, a Novel Vertebrate Germ Plasm Component, Is Required for Zebrafish Primordial Germ Cell Migration and Survival**

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Summary

In most animals, primordial germ cell (PGC) specification and development depend on maternally provided cytoplasmic determinants that constitute the so-called germ plasm [1–5]. Little is known about the role of germ plasm in vertebrate germ cell development, and its molecular mode of action remains elusive. While PGC specification in mammals occurs via different mechanisms [6], several germ plasm components required for early PGC development in lower organisms are expressed in mammalian germ cells after their migration to the gonad and are involved in gametogenesis [7, 8]. Here we show that the RNA of *dead end*, encoding a novel putative RNA binding protein, is a component of the germ plasm in zebrafish and is specifically expressed in PGCs throughout embryogenesis; Dead End protein is localized to perinuclear germ granules within PGCs. Knockdown of *dead end* blocks confinement of PGCs to the deep blastoderm shortly after their specification and results in failure of PGCs to exhibit motile behavior and to actively migrate thereafter. PGCs subsequently die, while somatic development is not effected. We have identified *dead end* orthologs in other vertebrates including *Xenopus*, mouse, and chick, where they are expressed in germ plasm and germ-line cells, suggesting a role in germ-line development in these organisms as well.

Results and Discussion

Zebrafish *dead end* RNA Is a Component of the Germ Plasm and Is Specifically Expressed in Primordial Germ Cells

In a large-scale whole-mount in situ screen for genes expressed in zebrafish PGCs, we identified a novel gene, *dead end* (*dnd*), which is specifically expressed in germ plasm and primordial germ cells. Shortly after fertilization, maternal *dnd* RNA is present in numerous granules distributed throughout the cortex of the one-cell stage embryo (Figure 1A). During the later phase of the first cell cycle, these granules disappear from the animal pole of the embryo and concentrate at the vegetal part of the blastomere (Figure 1B). Subsequently, *dnd* RNA is enriched at the distal parts of the first two cleavage furrows (Figure 1C). This expression pattern is similar to that of *vasa* RNA, which is known to reside within the zebrafish germ plasm [9, 10].

During early cleavage stages, the zebrafish germ plasm is incorporated into four blastomeres, which asymmetrically distribute it to only one daughter during subsequent cell divisions [10]. At the late blastula stage (4 hr postfertilization [hpf], late sphere stage), PGC specification occurs, and as a result in subsequent divisions the germ plasm is symmetrically distributed to both daughter cells that appear to be committed to the germ-line [10]. It has been previously shown that a substantial fraction of maternal *vasa* RNA is not incorporated into the PGCs and remains detectable in somatic cells up to early gastrulation stages (6 hpf) [9–11]. An even greater fraction of *dnd* RNA is present in future somatic cells up to early blastula stages (3.5 hpf, Figures 1D and 1E). Shortly thereafter, however, the somatic RNA disappears, and by 4.3 hpf *dnd* is expressed exclusively in the PGCs (Figure 1F). *dnd* continues to be expressed in PGCs during their migration (Figure 1G) and as they reach the position of the presumptive gonad (arrow in Figure 1H). At 5 days post fertilization (dpf), *dnd* RNA is still detectable in the PGCs, albeit at a lower level (data not shown). Consistent with the spatial distribution of the mRNA, Northern blot analysis revealed rapid degradation of the maternally provided *dnd* RNA just after PGC specification at 4 hpf (sphere stage) (Figure 1I). Low levels of RNA persist during embryogenesis, which presumably corresponds to expression of the gene exclusively in the PGCs. In addition to the tight control of maternal *dnd* RNA stability, *dead end* transcripts appear to be translationally repressed, as revealed by the ability of the *dnd* 3' untranslated region (UTR) to inhibit translation of a green fluorescent protein (GFP) open reading frame upon injection of fusion RNAs into early embryos (data not shown). Such control mechanisms suggest that expression of Dead end in somatic cells may be deleterious for embryonic development. Indeed, overexpression of high doses of Dead end in the whole embryo resulted in strong inhibition of somatic development beyond gastrulation, while overexpression specifically in

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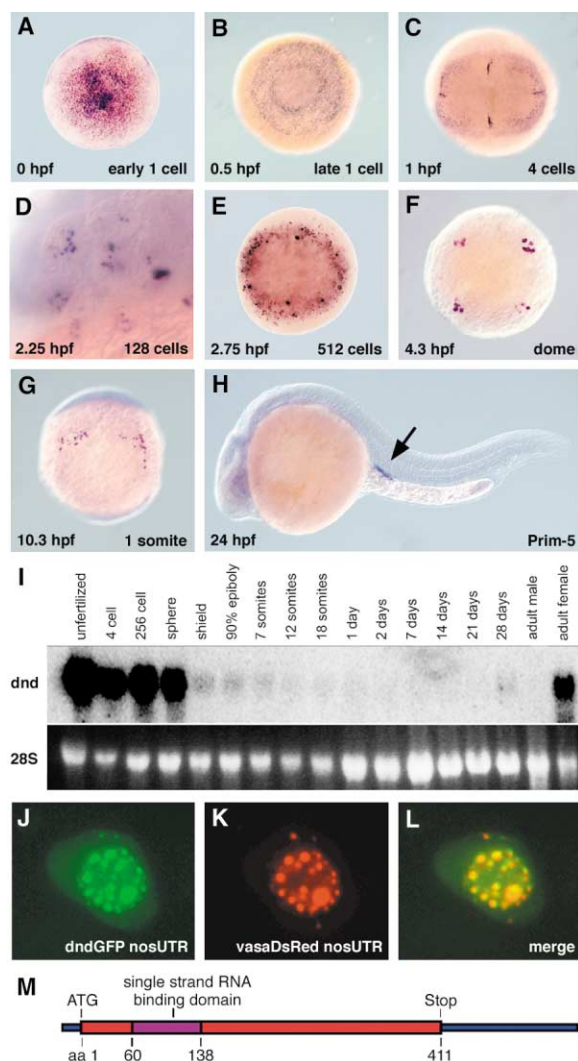


Figure 1. Zebrafish *dead end* Is Expressed in Germ Plasm and Primordial Germ Cells and Encodes a Putative RNA Binding Protein
(A–H) Whole-mount in situ hybridizations of embryos with *dnd* antisense RNA probe at the indicated stages. (D) is a high-magnification view of ectopic *dnd*-RNA containing granules in somatic cells. Note that *dnd* is exclusively expressed in PGCs from dome stage onward (F). (I) Northern blot analysis using *dnd* as probe.
(J–L) Fluorescent pictures taken at the 10-somite stage of a PGC in an embryo coinjected with 100 pg *dndGFP nos1-3'UTR* (green channel in J) and *vasaDsRed nos1-3'UTR* (red channel in K). The merge picture in (L) shows that Vasa and Dead end proteins colocalize to perinuclear germ plasm granules.
(M) Cartoon of the zebrafish *dnd* cDNA depicting the ORF of 411 amino acids in red and the single-strand RNA binding domain (Prosite profile PS50102) in purple.

the PGCs had no effect on somatic development or PGC number (data not shown).

Dead End Is a Novel Putative RNA Binding Protein which Is Localized to Perinuclear Germ Granules

The zebrafish *dead end* cDNA encodes a protein of 411 amino acids containing a putative single-strand RNA binding domain (Prosite database profile PS50102) in its N-terminal half (Figure 1M). This RNA binding motif

is found in a large variety of RNA binding proteins such as heterogeneous nuclear ribonucleoproteins (hnRNPs), small nuclear ribonucleoproteins (snRNPs), and other pre-RNA and mRNA-associated proteins.

To determine the subcellular localization of the Dead end protein, a Dead end-GFP fusion protein was expressed in the PGCs. As shown in Figures 1J–1L, the Dead end-GFP protein is localized to perinuclear germ granules that also contain a Vasa-DsRed fusion protein in mid-somitogenesis stage embryos. Thus, Dead end is localized to the same cellular structure where other zebrafish germline-specific proteins (e.g., Vasa and Nanos) are found. Similar germ granules that contain multiple putative RNA binding proteins are found in germline cells of many organisms, and intriguingly, in *C. elegans* these structures are found in association with nuclear pores [12]. It has been proposed that germ granules regulate gene expression in PGCs posttranscriptionally, by modifying mRNA transport, stability, and translation [3, 13]. The existence of a putative RNA binding domain in the Dead end protein and its localization to germ granules make it likely that *dnd* functions in one of these processes.

Dead End Is Required for PGC Migration

In most species, PGCs migrate from the site at which they are specified toward the developing gonad [14]. Zebrafish PGCs start to migrate shortly before gastrulation and follow six distinct migration steps before arriving at the region where the gonad develops [9, 15–17]. As migration initiates, most of the PGCs are confined to the deep layers of the blastoderm and are found in close proximity to the yolk syncytial layer (YSL) (4.3 hpf, dome stage, Figure 2A). However, inhibition of *dead end* translation by injection of *dnd*-specific morpholino antisense oligonucleotides (*dnd*-MO) resulted in detachment of PGCs from the YSL (arrows in Figure 2B). The abnormal positioning of the PGCs at 4.3 hpf is the first phenotype observed as a consequence of *dnd* knockdown; earlier, at 3.8 hpf (early sphere), as PGCs are specified, no significant difference in the distribution or number of cells expressing the PGC marker *nanos-1* (*nos-1*) was detected between controls and *dnd*-MO-injected embryos (Figure 2C). Thus, it appears that inhibition of *dead end* translation in early embryos does not affect PGC specification and initial confinement of PGC-precursors to the deep blastoderm. However, after PGC specification, an increasingly severe PGC localization phenotype was observed from 4.7 to 7 hpf, during which time a large number of ectopic PGCs was found in the outermost cell layer in *dnd* knockdown embryos (Figure 2C). This finding suggests that the confinement of the PGCs to the deep cell layers is actively regulated and represents a previously unknown property of wild-type PGCs, which is dependent on *dnd* function. The earliest step of active PGC migration in zebrafish described in preceding reports occurs during early gastrulation, when PGCs located at the dorsal side of the embryo vacate the dorsal midline [15]. In a population of *dnd*-MO-injected embryos, 14% of all PGCs ($n = 305$ cells) are found in a dorsal sector spanning 60° of the embryo's circumference at 7 hpf, as opposed to 1% in control-

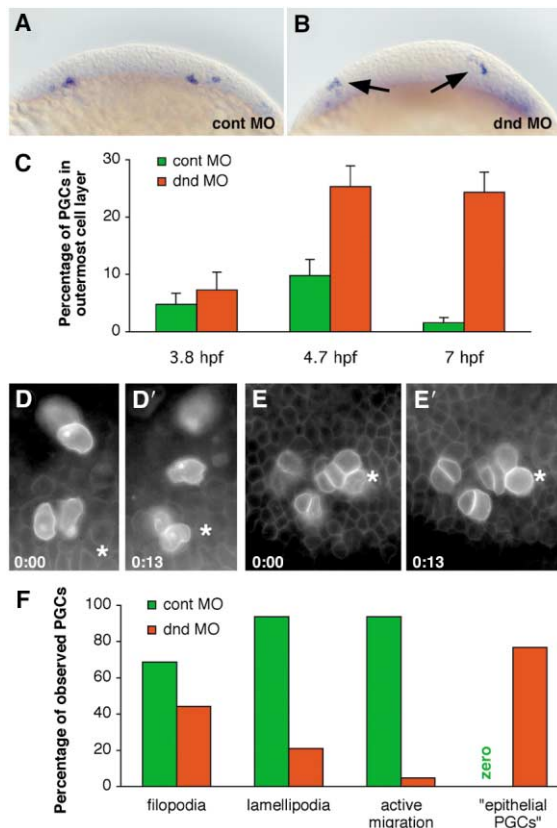


Figure 2. *dead end* Is Required for Zebrafish PGC Migration

(A–C) *Dead end* knockdown results in detachment of PGCs from the YSL. (A and B) Optical cross-sections of embryos at dome stage (4.3 hpf) show that *nos-1*-positive PGCs are confined to the deep blastoderm in embryos injected with 1400 pg control MO (A), but not *dnd*-MO-injected (B) embryos. (C) The percentage of *nos-1*-positive PGCs found in the outermost cell layer of embryos at the indicated time points in embryos injected with 400 pg control (green) or *dnd* MO (red). In 91% of *dnd*-MO-injected and 25% of control-MO-injected embryos, at least 1 PGC (of an average of 5.2 for *dnd* MO) was found in the outermost cell layer at 7 hpf.

(D and E) Frames taken at the indicated times from time-lapse movies (Movies 1 and 2 in the Supplemental Data) showing PGCs during early somitogenesis labeled with membrane-localized GFP in embryos injected with 400 pg control MO (D and D') or *dnd* MO (E and E'). The position of a somatic cell is depicted by an asterisk in both movies. Note that control PGCs actively migrate as individual cells relative to their somatic neighbors (D and D'). In contrast, *dnd* knockdown-PGCs do not actively migrate and form close cell-cell contacts (E and E').

(F) Quantification of PGC behavior at early somitogenesis in embryos injected with 400 pg control MO (green) or *dnd* MO (red). Short timelapse movies of PGCs labeled by membrane-localized GFP were analyzed for the presence of filopodia and lamellipodia on PGCs, active migration of PGCs relative to somatic neighbors, and for close contacts between PGCs that remained stable throughout the movie ("epithelial" appearance). The percentage of observed PGCs exhibiting these features is shown. $n = 16$ control PGCs, 43 *dnd* knockdown-PGCs.

MO-injected embryos ($n = 237$, $p = 0.02$), and this phenotype is observed in 64% of the embryos injected with the *dead end* morpholino ($n = 11$). Given that in a population of embryos the fraction of cells that perform this migration step is about 17% and that it is performed in

only two-thirds of the embryos, the observed phenotype is fully penetrant and PGC migration away from the dorsal midline appears to be completely blocked by knockdown of *Dead end* (for details, see the Supplemental Data available with this article online). Together, these data show that *Dead end* function is required for one of the first manifestations of primordial germ cell behavior, namely carrying out specific migration steps during early embryonic development. Importantly, despite the strong defects in PGC migration, *dnd* knockdown did not affect somatic development as judged by the normal morphology of the embryos, the normal expression of various marker genes, and by the survival of the treated fish to adulthood. In addition, at pre- and early gastrulation stages, the PGCs in morpholino-treated embryos express characteristic PGC markers such as *nos-1* (Figure 2B) and *vasa* (not shown) and they proliferate at wild-type rates (see below), supporting the view that the early migration defect does not reflect a secondary effect of abnormal PGC specification. Nevertheless, it is formally possible that some aspects of PGC specification that were not revealed by the analysis described above contribute to the migration phenotype.

To determine the cellular basis for the migration phenotype described above, we examined *Dead end*-depleted PGCs in live embryos and compared their behavior to that in control embryos. Farnesylated *EGFP-nos1-3'UTR* RNA was injected into 1 cell stage embryos to label the membrane of PGCs. The ability to direct PGC-specific expression of proteins in *Dead end*-depleted embryos using this method [18] lends further support to the notion that specification and early maintenance of PGC fate do not require *Dead end*. However, in contrast to GFP-labeled PGCs in control-MO-injected embryos, which exhibit active migration relative to their somatic neighbors (Figures 2D and 2D'; see Movie 1 in the Supplemental Data), there is no evidence for active PGC migration in *dnd* knockdown embryos from late gastrulation throughout somitogenesis (Figures 2E, 2E', and 2F; see Movie 2 in the Supplemental Data). Furthermore, unlike control PGCs, which migrate as individual cells, *dnd* knockdown PGCs often remain in groups of cells that maintain close cell-cell contact (Figures 2E, 2E', and 2F). Nevertheless, in most cases *Dead end*-depleted PGCs did show some morphological features of motile cells. Specifically, while formation of lamellipodia was strongly reduced, the number of PGCs extending filopodia was less affected compared to controls (Figure 2F). Thus, functional *Dead end* is required for one of the fundamental properties of PGCs, which is their ability to migrate. To the best of our knowledge, this is the first description of a gene product required for the transition of PGCs from stationary to motile cells. It will therefore be interesting to identify the proteins and RNAs with which *Dead end* interacts, as these may provide a hint regarding the molecular basis of the control of migratory cell behavior. Importantly, the *dead end* knockdown phenotype is strikingly different from that described for PGCs lacking CXCR4b signaling [17, 19]. While showing severe defects in directional migration, PGCs lacking CXCR4b are motile and exhibit active migration relative to their somatic neighbors [17, 19].

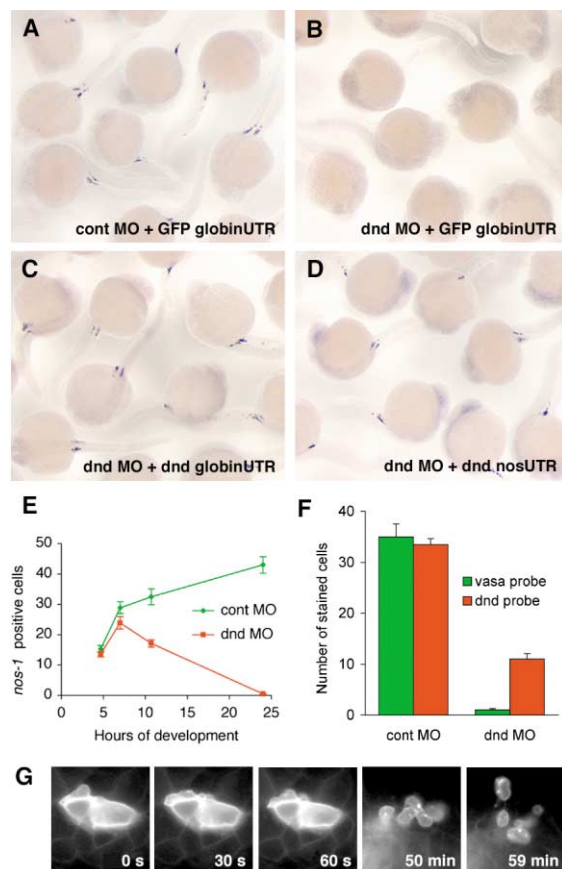


Figure 3. Zebrafish *dead end* Is Essential for PGC Survival

(A–D) *nos-1* expression at 24 hpf in embryos injected with the indicated MOs (200 pg) plus RNAs (1.7×10^{-16} moles). Expression of *nos-1* is lost in *dnd* MO plus control *GFP globinUTR* RNA injected embryos (B) but rescued to levels seen in control-MO-injected embryos (A) by coinjection of a MO-insensitive RNA coding for *dnd* that is stable in the whole embryo (*dnd globinUTR*) (C) or targeted to the PGCs by the *nos1* 3' UTR (*dnd nos1-3' UTR*) (D).

(E) Time course of loss of *nos-1* expression. Average numbers of *nos-1*-RNA-positive PGCs in embryos injected with 1 ng of control MO (green) or *dnd* MO (red) plotted against developmental time. $n \geq 12$ embryos for each data point, error bars represent the SEM. (F) Average numbers of cells expressing *vasa* RNA (green) or *dead end* RNA (red) in embryos injected with 200 pg control MO or *dnd* MO at 22 hpf. Note that the probes detect the same number of PGCs in controls, but in *dnd*-MO-injected embryos some PGCs have lost *vasa* while they continue to express *dnd*. $n \geq 9$ embryos for each data point, error bars represent the SEM.

(G) Frames taken at the indicated times from a time-lapse movie (Movie 3 in the Supplemental Data) that starts at the 2-somite stage and shows two closely attached PGCs labeled by membrane-localized GFP in an embryo injected with 400 pg *dnd* MO. Note the rapid membrane blebbing seen in the left PGC in the first three frames. In the last two frames one of the PGCs has fractionated into small cell fragments.

Zebrafish *dead end* Is Required for PGC Survival

To determine the fate of the PGCs in embryos in which *dead end* was knocked down, we followed the cells at later stages of development. Although, as mentioned above, a normal number of cells expressing PGC-specific markers was observed at early stages of development, no PGCs expressing *nos-1* or *vasa* RNA were observed by 24 hpf (Figures 3B and 3F). Similarly, while

PGCs in which *Dead end* was knocked down expressed GFP (Figure 2E), all of the GFP-positive PGCs disappeared by the end of the first day of development (data not shown). The migration defects and the loss of PGCs were fully reversed by restoring *Dead end* expression either in the whole embryo or specifically in the PGCs (Figures 3C and 3D). In the latter rescue experiment, injection of a morpholino-insensitive *dead end* RNA fused to the *nos1*-3' UTR reversed the phenotype at 24 hpf from 0.2 ± 0.2 cells per embryo ($n = 18$ *dnd*-MO-injected embryos, average \pm standard error of the mean) to 29.2 ± 1.2 ($n = 33$ *dnd*-MO, *dnd nos1*-3' UTR RNA injected embryos), which is similar to the number of PGCs in control-MO-injected embryos (33.5 ± 1.2 , $n = 33$). This experiment confirmed that the morpholino specifically inhibits *Dead end* function and indicates that *Dead end* activity is required within the PGCs themselves.

As determined on the basis of *nos-1* RNA expression, the initial number of PGCs in embryos injected with *dnd*-MO is similar to that in control embryos, and these cells divide at an almost normal rate until early gastrulation stages (6 hpf, Figure 3E). From this point on until the end of the first day of development, expression of *nos-1* was gradually lost. *vasa* RNA expression is lost at about the same rate as *nos-1*, but PGCs continue to express *dead end* RNA for several additional hours. Thus, while hardly any *vasa*-positive cells can be detected in *dnd*-MO-injected embryos at 22 hpf, numerous *dnd* positive cells are still present, all of them in ectopic positions (Figure 3F and data not shown). To determine the fate of these PGCs, we tracked individual GFP-labeled PGCs in live embryos. We found that in contrast to control-MO-injected embryos, all PGCs in *dnd* knockdown embryos eventually exhibited the morphological hallmarks of apoptotic cells [20], including membrane blebbing and fractionation into small bodies (Figure 3G and Movie 3 in the Supplemental Data). The initiation of PGC death in *dnd* knockdown embryos occurred about 6 hr after the corresponding start of loss of *nos-1* expression (data not shown). Although we cannot exclude the possibility that a small number of PGCs assumes a different fate, we favor the conclusion that PGCs lacking functional *Dead end* lose expression of characteristic genes and later die. While inhibiting the translation of *dnd* transcripts did not affect PGC specification, it should be noted that injection of morpholinos into early embryos cannot interfere with the function of proteins deposited in the egg during oogenesis. Thus, it is possible that maternally provided *Dead end* protein plays a role in PGC specification.

The current understanding of the germline origin in zebrafish has been based on the expression of specific molecular markers, such as *vasa*, by cells that arrive at the region of the gonad and expression of the same markers later during gametogenesis. In *cxc4* morphants or mutants, the PGCs exhibit severe migration defects, yet a large proportion of adult *cxc4* mutants are fertile [19]. Therefore, there is no evidence that no other cells can potentially contribute to the germline during normal development, or when the number of *vasa*-expressing cells is reduced. To investigate this aspect, we raised control and *dnd*-MO-injected embryos to adulthood and

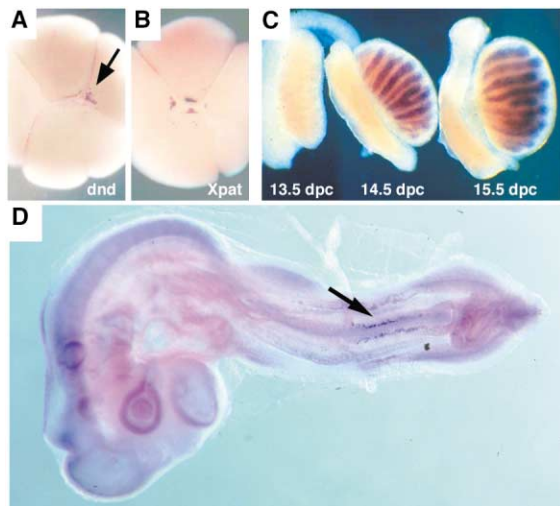


Figure 4. *dead end* Orthologs Are Expressed in Germ Plasm and Germ Cells in *Xenopus*, Mouse, and Chick

(A and B) Vegetal views of *Xenopus laevis* embryos at the 16-cell stage stained for expression of *dnd* (arrow in A) and *Xpat* (B) RNAs. Both are present in aggregates at the cleavage planes where the *Xenopus* germ plasm resides.

(C) Mouse gonads plus attached mesonephroi explanted from embryos at the indicated days post coitum (dpc) stained for expression of *dnd* RNA.

(D) Expression of chick *dnd* in PGCs (arrow) after their arrival at the region of the gonad (stage 18 according to [25]).

crossed them with wild-type fish; for unknown reasons, all adult *dnd* knockdown fish obtained turned out to be males. Remarkably, while on average 84% of the eggs were fertilized in crosses with control-MO-injected males (1333 of 1592 eggs), only 1% was fertilized by *dnd*-MO males (20 of 1486, all derived from one male). 14 of 15 *dnd*-MO-injected males did not fertilize a single egg in several crosses, while all 7 of the control-MO-injected males fertilized eggs at least in one cross. This strong correlation between loss of cells expressing PGC markers like *vasa* and *nos-1* and sterility further corroborates the notion that the only cells capable of populating the germline in the zebrafish gonad are the cells expressing these markers at earlier stages. Indeed, this finding allows for the complete replacement of the germline in *dnd*-MO-injected embryos with that of genetically marked donor embryos by transplantation, an achievement that significantly simplifies the production of maternal effect mutants [21].

dead end Is Conserved in Other Vertebrate Species

While *dead end* function is clearly crucial for proper development of zebrafish PGCs, no homologous proteins have been described so far. However, we could identify ESTs and genomic sequences encoding closely related genes in *Xenopus laevis*, chick, mouse, and human (Figure S1). These *dead end* orthologs are expressed in the germ plasm (of *Xenopus*) and in germ cells (of mouse and chick), implying that they might play a role in germline development in these organisms, too. In *Xenopus* maternal *dead end* RNA is present along the cleavage planes at the vegetal pole of early cleavage stage embryos (arrow in Figure 4A) in a pattern very

similar to that of *Xpat*, a germ plasm component in that organism (Figure 4B) [22]. Expression of *dead end* in the mouse also appeared to be restricted to germ cells, as seen at 14.5 dpc, where expression is very strong within the cords (Figure 4C). This expression pattern is reminiscent of the expression pattern of the mouse *vasa* homolog (*mvh*), which is expressed in the PGCs as they arrive at the region of the gonad [23]. The chick *dead end* was expressed in the PGCs, in a similar manner to that of chick *vasa*, and could be identified in those cells before, during, and after their arrival at the gonad (Figure 4D and data not shown). Therefore, based on the similarity in sequence and expression pattern, it is likely that *dead end* plays a general role in germline development in other vertebrates, as well.

Interestingly, we failed to identify *dead end* orthologs in the fully sequenced genomes of *Drosophila melanogaster* and *Caenorhabditis elegans*. Specifically, proteins from these invertebrates showing similarity to *Dead end* orthologs show significantly higher degree of similarity to other proteins in vertebrates. This raises the possibility that *dead end* is the first vertebrate-specific germ plasm component known to be essential for germ cell development.

Conclusions

Knockdown of zebrafish *dead end* results in failure of PGCs to express germline-specific properties, most notably migration, and leads to subsequent PGC death. While the requirement of germ plasm components for the specification and migration phases of germ cell development differs between organisms, germ plasm and at least some of its components appear to be universally required for gametogenesis. Since *dead end* is expressed in postmigratory germ cells in vertebrates, it will be interesting to test which role it plays in this phase of germ cell development. Given the apparent absence of *dead end* orthologs in invertebrate model organisms, identification of its interacting partners should reveal whether it fulfills the role of another molecule in invertebrates or perhaps is part of a vertebrate-specific pathway in germ cell development.

Experimental Procedures

Cloning of *dead end*

The full-length sequence of zebrafish *dnd* (GenBank accession number AY225448) was determined by 3' and 5' RACE and alignment with EST sequences derived from GenBank. The mouse open reading frame (ORF) (accession number AY321066) and the human ORF (accession number AY321065) *dnd* cDNA sequences were predicted from genomic and EST sequences based on homology with zebrafish *dnd*. The structure of the mouse cDNA was confirmed by PCR-amplifying and sequencing a clone from adult testis cDNA (mouse strain C57BL/6J). *dnd* cDNAs recently predicted from the mouse genome by the NCBI annotation project (GenBank accession number XM 140262) and the mammalian gene collection (accession number BC034897) contain the first intron and likely are artifacts, since their ORFs start only at the seventh ATG. The sequence of the full-length *Xenopus dnd* ORF (GenBank accession number AY321494) was obtained by sequencing EST db33c06. The chick *dnd* EST used as template for in situ probe synthesis has the GenBank accession number BM440036.

Injections and Constructs Used

The GFP variant mmGFP-5 was used in all constructs [24]. Capped sense RNAs were synthesized in vitro using the Message Machine

kit (Ambion) and injected at the quantities indicated in the figure legends according to standard procedures.

dndGFP nos1-3' UTR (#516): fusion protein of zebrafish Dead end with GFP at the C terminus; RNA contains the zebrafish *nos-1* 3' UTR.

vasaDsRed nos1-3' UTR (#363): N-terminal 369 amino acids of zebrafish Vasa, which are sufficient for localization to perinuclear granules [11], fused to DsRed (Clontech) at the C terminus; RNA contains the zebrafish *nos1* 3' UTR.

GFP globinUTR (#297): GFP flanked by *Xenopus globin* 5' and 3' UTRs.

dnd globinUTR (#487): zebrafish *dnd* ORF containing 5 nucleotide changes in the *dnd* morpholino binding site that do not alter the encoded protein sequence; RNA contains the *Xenopus globin* 3' UTR.

dnd nos1-3' UTR (#495): identical to *dnd globinUTR* except that it contains the zebrafish *nos1* 3' UTR.

Dead end Knockdown

The *dead end* morpholino antisense oligonucleotide (*dnd* MO, 5'-GCTGGGCATCCATGTCTCCGACCAT-3') and the standard control MO were obtained from Genetools, Philomath, OR. PGC loss at 24 hpf was dependent on the amount of MO injected per embryo: as little as 10 pg decreased numbers of *nos-1* positive cells significantly, and concentrations above 200 pg resulted in complete loss of PGCs. Throughout this study, 200 to 1400 pg were injected.

In Vivo Observation of PGCs

PGCs were labeled in live embryos by injection of 40 pg of *EGFPF-nos1-3' UTR* as described in [16].

Supplemental Data

Supplemental Data including additional Experimental Procedures, a figure, and three movies are available at <http://www.current-biology.com/cgi/content/full/13/16/1429/DC1>.

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